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Citation for published version:

Hurtado gonzalez, P, Anderson, RA, Macdonald, J, van den Driesche, S, Kilcoyne, K, Jorgensen, A, McKinnell, C, Macpherson, S, Sharpe, R & Mitchell, RT 2018, 'Effects of exposure to acetaminophen and ibuprofen on fetal germ cell development in both sexes in rodent and human using multiple experimental systems', *Environmental Health Perspectives*. <https://doi.org/10.1289/EHP2307>

Digital Object Identifier (DOI):

[10.1289/EHP2307](https://doi.org/10.1289/EHP2307)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Publisher's PDF, also known as Version of record

Published In:

Environmental Health Perspectives

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Effects of Exposure to Acetaminophen and Ibuprofen on Fetal Germ Cell Development in Both Sexes in Rodent and Human Using Multiple Experimental Systems

Pablo Hurtado-Gonzalez,¹ Richard A. Anderson,¹ Joni Macdonald,¹ Sander van den Driesche,² Karen Kilcoyne,¹ Anne Jørgensen,³ Chris McKinnell,¹ Sheila Macpherson,¹ Richard M. Sharpe,¹ and Rod T. Mitchell¹

¹Medical Research Council (MRC) Centre for Reproductive Health, Queen's Medical Research Institute, University of Edinburgh, Edinburgh, UK

²Centre for Discovery Brain Sciences, Biomedical Sciences, University of Edinburgh, Edinburgh, UK

³Department of Growth and Reproduction, Copenhagen University Hospital (Rigshospitalet), Copenhagen, Denmark

BACKGROUND: Analgesic exposure during pregnancy may affect aspects of fetal gonadal development that are targeted by endocrine disruptors.

OBJECTIVES: We investigated whether therapeutically relevant doses of acetaminophen and ibuprofen affect germ cell (GC) development in human fetal testes/ovaries using *in vitro* and xenograft approaches.

METHODS: First-trimester human fetal testes/ovaries were cultured and exposed to acetaminophen or ibuprofen (7 d). Second-trimester human fetal testes were xenografted into mice and exposed to acetaminophen (1 or 7 d), or ibuprofen (7 d). To determine mechanism of action, a human GC tumor–derived cell line (NTera2) exhibiting fetal GC characteristics was used in addition to *in vitro* and *in vivo* rat models.

RESULTS AND DISCUSSION: Gonocyte (TFAP2C⁺) number was reduced relative to controls in first-trimester human fetal testes exposed *in vitro* to acetaminophen (–28%) or ibuprofen (–22%) and also in ovaries exposed to acetaminophen (–43%) or ibuprofen (–49%). Acetaminophen exposure reduced gonocyte number by 17% and 30% in xenografted second-trimester human fetal testes after treatment of host mice for 1 or 7 d, respectively. NTera2 cell number was reduced following exposure to either analgesic or prostaglandin E₂ (PGE₂) receptor antagonists, whereas PGE₂ agonists prevented acetaminophen-induced reduction in NTera2 cell number. Expression of GC pluripotency genes, and genes that regulate DNA/histone methylation, also differed from controls following analgesic and PGE₂ receptor antagonist exposures. Gene expression changes were observed in rat fetal testis/ovary cultures and after *in vivo* acetaminophen exposure of pregnant rats. For example, expression of the epigenetic regulator *TET1*, was increased following exposure to acetaminophen in human NTera2 cells, rat fetal testis/ovary cultures, and in fetal testes and ovaries after *in vivo* exposure of pregnant rats, indicating translatability across experimental models and species.

CONCLUSIONS: Our results demonstrate evidence of PGE₂-mediated effects of acetaminophen and ibuprofen on GC/NTera2 cells, which raises concerns about analgesic use during human pregnancy that warrant further investigation. <https://doi.org/10.1289/EHP2307>

Introduction

Epidemiological studies support the view that maternal exposure to certain environmental chemicals with endocrine-disrupting potential may be associated with adverse effects on reproductive development of the resulting offspring, including androgen-dependent processes in males (Skakkebaek et al. 2016). More recently, experimental animal evidence suggests that *in utero* exposures to endocrine-disrupting chemicals could have intergenerational effects via epigenetic changes to fetal germ cells (Lane et al. 2015; Braun et al. 2017). In contrast with unintentional exposure to low levels of environmental chemicals, pregnant women may be intentionally exposed to relatively high doses of pharmaceuticals—if medications have reproductive developmental effects, and their use is associated with environmental exposures, they could confound associations between *in utero* environmental chemical exposures and developmental outcomes in human observational studies. In this context, data collected from pregnant women in the United States (Werler et al. 2005), France (Philippat et al. 2011), and Denmark (Jensen et al. 2010) during the late 1990s to mid-2000s

indicated that the majority (55% in Denmark, 70–76% in the United States, 89% in France) used an analgesic at least once during pregnancy, with most (47–66%) reporting use of acetaminophen (paracetamol) and 5–15% reporting use of ibuprofen (a nonsteroidal anti-inflammatory drug; NSAID), both of which are available without medical prescription (Campbell et al. 2016; Werler et al. 2005). Acetaminophen and NSAIDs are able to cross the placenta into the fetal circulation and as a result have the potential to affect fetal development (Alano et al. 2001; Naga Rani et al. 1989; Nitsche et al. 2017; Weigand et al. 1984).

Epidemiological studies have reported some evidence of associations between analgesic use during pregnancy and cryptorchidism in sons, though findings have been inconsistent within and among different study populations (Berkowitz and Lapinski 1996; Jensen et al. 2010; Kristensen et al. 2011; Philippat et al. 2011; Snijder et al. 2012). Testicular descent is primarily under the influence of testosterone produced by the Leydig cells of the fetal testis, and experimental studies have shown that the analgesics, acetaminophen, ibuprofen, and aspirin can all reduce testosterone production by the fetal testis in the rat (Kristensen et al. 2011, 2012; van den Driesche et al. 2015). A recent study using a xenograft model of human fetal testis tissue collected between 14–20 gestational weeks reported that prolonged acetaminophen exposure at a human-relevant dose (20 mg/kg three times per day for 7 d) decreased plasma testosterone levels in xenografted mice (van den Driesche et al. 2015). In addition, treatment of pregnant rats with a comparable acetaminophen dose suppressed the expression of specific steroidogenic enzymes (*Cyp11a1* and *CYP17a1*) in rat fetal testes.

Recent studies have investigated analgesic exposure during pregnancy in relation to fetal germ cell (GC) development and later reproductive function in rodents (Dean et al. 2016; Holm et al. 2016). Modifications in fetal GC might not only affect the future reproductive potential of the exposed fetus, but could

Address correspondence to R.T. Mitchell, MRC Centre for Reproductive Health, The University of Edinburgh, The Queen's Medical Research Institute, 47 Little France Crescent, Edinburgh EH16 4TJ, Scotland, UK. Telephone: 44 131 242 6470. Email: rod.mitchell@ed.ac.uk

Supplemental Material is available online (<https://doi.org/10.1289/EHP2307>).

The authors declare they have no actual or potential competing financial interests.

Received 7 June 2017; Revised 11 February 2018; Accepted 23 February 2018; Published 16 April 2018.

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potentially affect future generations (intergenerational effects). In an experimental rat study, gestational exposures to indomethacin or acetaminophen (single daily doses of 0.8 and 350 mg/kg, respectively) were associated with a 40–50% reduction in fetal ovary GC number and significant reductions in ovary weight and fertility in adult Filial 1 (F1) females, whereas exposure to indomethacin (an NSAID) was associated with a 43% reduction in fetal testes GC number (Dean et al. 2016). Moreover, fetal exposure to either analgesic was associated with abnormal ovarian development and function in the second generation, independent of the sex of the exposed F1 parent, suggesting an intergenerational effect transmitted via both paternal and maternal lines that was hypothesized to be a consequence of epigenetic changes to the GCs (Dean et al. 2016).

One mechanistic pathway that potentially links analgesics and epigenetic alterations is the prostaglandin (PG) pathway, through which acetaminophen, indomethacin, and ibuprofen can all act. Somatic and germ cells in the fetal gonads of both sexes in rats and humans are sources and targets of PGs because they express the PG-synthesizing COX-2 enzyme and PGE receptors (Bayne et al. 2009; Dean et al. 2016). Studies have shown that PGE₂ exerts a range of effects on normal and cancerous cells, *in vivo* and *in vitro* (Wang and Dubois 2006), including alterations in cell proliferation (Yun et al. 2009) and stem cell pluripotency (Wang et al. 2013; Yun et al. 2012). PGE₂-induced changes in DNA and histone methylation are also described and reported to be mediated by altered expression of key epigenetic regulatory factors including DNA methyltransferases (DNMT3a and b) and enhancer of zeste homolog 2 (EZH2) (Arosh et al. 2015; Venza et al. 2012; Xia et al. 2012).

For the present study, we used a combination of approaches, including culture and xenografting of human fetal gonads, NTera2 cells, culture, and pregnancy studies in rats, to investigate the effects of acetaminophen and ibuprofen exposures at human therapeutically relevant levels on GC number and pluripotency in the human fetal testis and ovary, and to determine whether effects involved the PGE₂ pathway and altered the expression of key epigenetic regulatory factors.

Materials and Methods

Study Design

We aimed to determine whether exposure to acetaminophen or ibuprofen affects GC number in fetal human gonads and to examine the potential mechanisms involved. The effect of common analgesics was studied in a variety of model systems *in vitro* and *in vivo*. The models and their specific treatment regimens are shown in Figure 1. Effects of exposure were studied in human fetal gonads taken from different stages of pregnancy using hanging drop culture (first-trimester testes and ovaries) and a xenograft system (second-trimester testes). Sample sizes (minimum *n* = 3) were based on those required to achieve statistical significance in previous studies using the same methodology (Mitchell et al. 2012, 2013), and analgesic dose–response analyses were not performed because of the restricted availability of human fetal samples.

The analgesic concentrations used in cultures (10 μM; Figure 1A) were based on previous studies (Janssen and Venema 1985; Kristensen et al. 2012; Mazaud-Guittot et al. 2013) and are relevant to peak serum concentrations in humans following therapeutic analgesic exposure reported as 1.37 μM for acetaminophen 48 min after exposure (Rayburn et al. 1986) and 2.5 μM for ibuprofen 1 h after exposure (Janssen and Venema 1985). To compare the effects of treatment versus vehicle in xenografts for each individual human fetal testis (Figure 1B), we grafted tissue from each

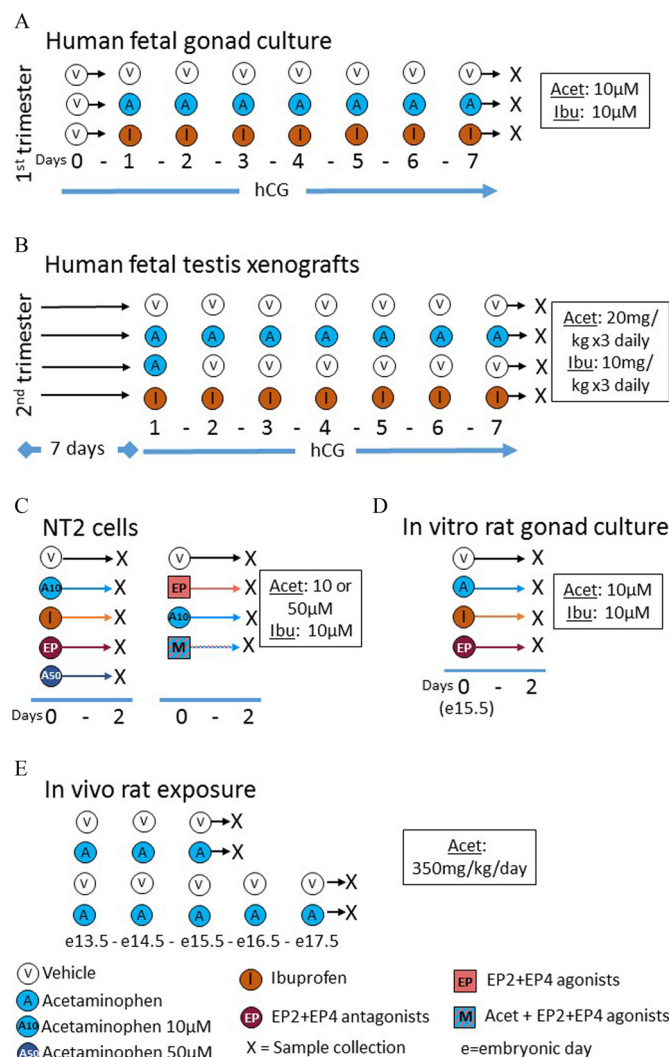


Figure 1. Exposure/treatment regimens and the different investigative models used in the present studies. Each panel shows a different experimental model together with the exposure/treatment (colored circles) regimen employed, including timescale. (A) Hanging drop culture of first-trimester human fetal testes and ovaries. Medium was supplemented with hCG (0.1 U/mL daily). (B) Xenografts of second-trimester human fetal testes grafted into nude mice. Note experimental treatments commenced 7 d after grafting in order to allow vascularization of the grafts. hCG (20 IU) was administered to host mice by subcutaneous injections every 72 h. (C) NTera2 (testicular germ cell tumor cell line) cell culture. Tissue cultured for 2 d (days 0–2) followed by analysis (indicated by “X”). (D) Hanging drop culture of fetal, embryonic day 15.5 (e15.5), rat testes and ovaries. Tissue cultured for 2 d (days 0–2) followed by analysis (indicated by “X”). (E) *In vivo* exposure of fetal rat testes and ovaries by treatment of pregnant rats from e13.5 until e15.5 or e17.5. The order of panels corresponds with the order of models presented in the “Results” section. Note: Acet, acetaminophen; hCG, human chorionic gonadotropin; Ibu, ibuprofen.

fetus into three to six replicate host mice and randomly allocated these to receive either acetaminophen/ibuprofen or vehicle treatment. For the xenograft studies, human-relevant oral doses administered to host mice were based on the UK maximum recommended daily dosing of acetaminophen (20 mg/kg three times daily) and ibuprofen (10 mg/kg three times daily) (<https://bnf.nice.org.uk/drug/paracetamol.html> and <https://bnf.nice.org.uk/drug/ibuprofen.html>). Previous studies have shown that this dose of acetaminophen produces a considerably lower plasma concentration in host mice (1 h post-dose) than that shown in humans

(0.94 µg/mL vs. 20.8 µg/mL) (van den Driesche et al. 2015; Rayburn et al. 1986).

Experiments to determine the mechanism of analgesic action used a GC tumor-derived cell line (NTera2), and end points evaluated included cell number, proliferation status, and expression of pluripotency markers and epigenetic regulators (Figure 1C). In addition, we used hanging drop cultures of rat fetal gonads to investigate whether similar effects were found in a rat model (Figure 1D). For human fetal gonad cultures, analgesic doses were 10 µM for both acetaminophen and ibuprofen. For *in vivo* rat studies (Figure 1E), pregnant dams were randomly allocated to receive either acetaminophen (350 mg/kg per day) or vehicle via oral administration. We previously reported that serum levels in pregnant rats 1 h after oral dosing with 350 mg/kg per day were 49.4 ± 13.9 µg/mL and undetectable in controls (Dean et al. 2016).

Animals

All aspects of animal housing, management, and treatment conformed to UK Home Office guidelines. Wistar rats (Charles River) were housed under standard conditions and CD1 nude mice (Charles River) were housed in individually ventilated cages. All animals had free access to tap water and soy-free diet (SDS). All animals, including the mice used for xenotransplantation were handled with care to avoid unnecessary pain and suffering. For rat studies, timed matings were established by the presence of a vaginal plug, defined as embryonic day 0.5 (e0.5).

Human Fetal Gonad Hanging Drop Cultures

Culture of first-trimester human fetal testes [$n = 5$; 8–11 gestational weeks of age (GW)] and ovaries ($n = 3$ 9–11 GW) were as previously described (Jørgensen et al. 2015). For each fetus, the number of tissue pieces available and the allocation of tissue to each experiment and analysis were determined by the size of the starting material. Tissue pieces from both testes of each individual fetus were randomly allocated to treatment or control, making the fetus the experimental unit. In brief, gonads were cut into $\sim 1\text{-mm}^3$ pieces and cultured individually in hanging drops containing 30-µL Alpha-MEM (Lonza) supplemented with 10% fetal bovine serum (Life Technologies), 10-µL/mL penicillin/streptomycin (Sigma-Aldrich), 1% 200-mM L-glutamine (100X) (Gibco), 1% MEM NEAA nonessential amino acids (100X) (Gibco), 2% sodium pyruvate 100 mM (100X) (Gibco), and 1% insulin-transferrin-selenium (100X) (Gibco). Plates were incubated for 8 d at 37°C under 5% CO₂. The medium was changed daily. Samples were cultured with human chorionic gonadotropin (hCG) alone during the first day, and thereafter the medium was supplemented with hCG (0.1 U/mL; Pregnyl; Organon Laboratories) plus vehicle (DMSO) or analgesic (10-µM acetaminophen or 10-µM ibuprofen) for the remainder of the culture period (Figure 1). hCG was added in order to mimic the *in utero* environment. Following culture, tissue samples were fixed in Bouin's solution (Clin-Tech) for 2 h for subsequent fluorescence immunohistochemistry.

Human Fetal Testis Xenograft Studies

Previous attempts by our lab using human fetal ovarian tissue xenografts did not achieve good viability, therefore xenograft experiments were only performed with testis tissue. Second-trimester human fetal testis tissue was used for *ex vivo* xenograft studies because they provide a more physiological model to study gonad development and sufficient testis tissue to perform the xenografts (Mitchell et al. 2010). Fetal testes ($n = 10$; 14–17 GW) pieces ($\sim 1\text{ mm}^3$) were xenografted into castrate male CD1 nude (host) mice (4–6 wk of age; $n = 44$) as described previously (van

den Driesche et al. 2015). In brief, mice were castrated at least 2 wk before xenografting. Analgesia (Carprofen, Pfizer) was given in the drinking water for 3 d after castration. Four to six pieces of testis tissue were grafted subcutaneously under the dorsal skin of each host mice on either side of the midline. For each fetus, tissue pieces were grafted into 36 host mice that were randomly allocated to receive treatment or vehicle. Grafts were maintained for 7 d, in order to establish a blood supply, followed by 1 d (acetaminophen) or 7 d (acetaminophen, ibuprofen) of analgesic exposure (Figure 1B). Host mice also received subcutaneous injections of 20 IU hCG every 72 h to mimic the *in utero* environment (van den Driesche et al. 2015). Host mice received vehicle (corn oil), acetaminophen (20 mg/kg three times daily; Sigma-Aldrich) or ibuprofen (10 mg/kg three times daily; Sigma-Aldrich); both analgesics were suspended in corn oil and administered by gavage. Host mice were sacrificed by cervical dislocation and grafts were retrieved and weighed before fixation in Bouin's solution for subsequent fluorescence immunohistochemistry. Blocks (one to two per host mouse) were randomly selected for immunohistochemical analysis and quantification of GC number and proliferation as described below.

Cell Culture

NTera2 cells (gift from P. Andrews; passages 25–35) were used to study potential mechanisms of analgesic action on GC. We chose NTera2 cells, a pluripotent human embryonal carcinoma cell line derived from an adult testicular GC cancer patient because they express markers of fetal GC, such as TFAP2C and hence they are commonly used as a fetal GC model (Hoei-Hansen et al. 2004). For expansion, cells were cultured in DMEM medium (Life Technologies), supplemented with 10% fetal calf serum (Life Technologies), 1% penicillin/streptomycin (Sigma-Aldrich) and 1% L-glutamine (Gibco). Cell cultures were washed with phosphate-buffered saline (PBS) (Life technologies), and disaggregated with TRypLE™ Express (Gibco). Cells were cultured at 37°C under 5% CO₂ and split 1:2 or 1:4 for passage when confluent. Cell-conditioned media were regularly tested for mycoplasma infection by ELISA (mycoprobe™; R&D Systems®). For treatment, NTera2 cells (200,000/well) were cultured for 48 h at 37°C under 5% CO₂. The medium was supplemented with either vehicle (DMSO), 10- or 50-µM acetaminophen, 10-µM ibuprofen, or 10-µM L-161,982 (EP2 antagonist, Caymanchem) + 10-µM PF04418948 (EP4 antagonist, Caymanchem) (Figure 1C).

For analysis with EP2 + EP4 agonists, NTera2 cells (excluding vehicle) were first exposed to 10-µM Butaprost (EP2 agonist; Abcam) plus 10-nM CAY10598 (EP4 agonist; Abcam) for 4 h before the medium was changed to the subsequent treatment as follows: EP2 + EP4 agonists, 10-µM acetaminophen or the combination of 10-µM acetaminophen + EP2 + EP4 agonists (Figure 1C) and cultured for a further 44 h (total duration of culture: 48 h). After treatment, cells were either disaggregated with TRypLE for cell counting or protein analysis (ELISA), or collected for gene expression with Trizol (Life Technologies). All experiments were repeated three times, except the recovery experiment (using EP2 + EP2 agonists together with acetaminophen), which was repeated four times. Cell number (including both viable and dead cells) was determined using a NucleoCounter® (Chemometec) according to manufacturer's guidelines, which involved resuspension of disaggregated cells in lysis and stabilization buffers (Chemometec). Cell death was determined from the same samples by flow cytometry. Propidium iodide (75 µM; BD biosciences) was added to disaggregated cells to identify dead cells, which were quantified by flow cytometry (Flow Analyzer 5L LSR Fortessa; BD).

Rat Fetal Gonad Hanging Drop Cultures

Rat fetal (e15.5; $n = 13$ – 30 , from 20 litters) testes and ovaries were cut into $\sim 1\text{-mm}^3$ pieces and cultured as described above for human hanging drop cultures. Culture was for 48 h with either vehicle, acetaminophen (10 μM), ibuprofen (10 μM), or L-161,982 (EP2 antagonist; 10 μM) + PF0441848 (EP4 antagonist; 10 μM) (Figure 1D). Samples were then fixed in Bouin's solution for immunohistochemistry or snap-frozen for gene expression analysis.

Rat in vivo Pregnancy Studies

Pregnant female rats ($n = 11$) were administered acetaminophen (350 mg/kg per day by oral gavage) suspended in corn oil from e13.5 until the day before sampling (e15.5 or e17.5), as described previously (Dean et al. 2013, 2016) (Figure 1E). This dose equates to a human equivalent dose of ~ 60 mg/kg (i.e., typical daily consumption in humans) based on calculation of body surface area (Reagan-Shaw et al. 2008), and has been shown to be non-toxic, based on previous findings that this dose was not associated with a reduction in the weight of dams or their pups (van den Driesche et al. 2015). Control dams ($n = 10$) were administered corn oil according to the same regimen. After treatment, rats were sacrificed by cervical dislocation and fetal gonads were dissected and frozen for gene expression analysis.

Fluorescence Immunohistochemistry

Fluorescence immunostaining was used to identify Sertoli cells (SOX9; Merck, Millipore; catalog no. AB5535) and GC subpopulations, namely gonocytes (TFAP2C; Santa Cruz Biotechnology; catalog no. sc-12762) and prespermatogonia (MAGEA4; gift from G. Spagnoli). Proliferative GC were identified by co-immunostaining for Ki67 (Abcam; catalog no. ab16667), and apoptosis was studied using Cleaved caspase (Cell Signaling Technologies; catalog no. 9661). All samples from the same experiment were stained in the same run. Sections (5 μm) were dewaxed in xylene and rehydrated using graded ethanol series. Sections were then subjected to antigen retrieval by pressure-cooking in 0.01-M citrate buffer (pH 6.0) for 30 s at 125°C, followed by 20 min in the decloaking chamber; and then incubated in 3% (vol/vol) hydrogen peroxide (VWR chemicals) in methanol (Fisher Chemical). Sections were then washed with Tris-buffered saline [TBS: 0.05-M Tris, 0.85% NaCl (pH 7.4)] and incubated for 30 min with 20% chicken serum, 5% bovine serum albumin (BSA) (Sigma) in TBS to block nonspecific binding of the antibodies. Slides were then incubated with primary antibody, diluted in TBS, in a humidified chamber overnight at 4°C; followed by incubation with the appropriate secondary antibody IgG conjugated to horseradish peroxidase (HRP; Santa Cruz Biotechnology), at a concentration of 1:200 in TBS for 30 min at room temperature. Sections were then incubated for 10 min with Tyramide (TSA Plus Cyanine 3 System, PerkinElmer Life Sciences) according to the manufacturer's instructions.

For double/triple immunohistochemistry, for each subsequent primary antibody, sections underwent further antigen retrieval by boiling in 0.01-M citrate buffer (pH 6.0) for 2 min in a decloaking chamber, followed by blocking, overnight incubation with the primary antibody, and incubation with the appropriate secondary antibody and Tyramide, as described above. In some cases, this step was followed by a final incubation with the nuclear counterstain, Hoechst (Thermo Fisher), diluted 1:4,000 in TBS for 10 min. Slides were washed in TBS and mounted (Permafluor; Beckman Coulter). Images were acquired using a Provis microscope (Olympus Optical) fitted with a DCS330 digital camera (Eastman Kodak).

Quantification of GC Number and GC Proliferation in Human Fetal Gonads

Fetal testis sections (one per cultured or xenografted tissue piece) were co-immunostained for sex-determining region Y-box 9 (SOX9; Sertoli cells), transcription-factor activating enhancer-binding protein 2 (TFAP2C; gonocytes), and melanoma-associated antigen 4 (MAGEA4; prespermatogonia). The number of cells from each cell population was counted manually and the relevant area determined (using ZEN software; ZEISS) by a single observer blinded to treatment. Three different GC populations were quantified: total GC (TFAP2C⁺ + MAGEA4⁺), TFAP2C⁺ GC, and MAGEA4⁺ GC. Small pieces of fetal testicular and ovarian tissue were used for culture, so analysis of GC number had to take account of seminiferous cord area (first-trimester testis culture), Sertoli cell number (second-trimester testis xenografts), or ovarian area (first-trimester ovary culture), which varied between samples. The total number of TFAP2C⁺ and MAGEA4⁺ GC per section was expressed relative to the relevant area in micrometers squared or cell number in that section, such that ratios were determined for total GC (TFAP2C⁺ + MAGEA4⁺), TFAP2C⁺ GC and MAGEA4⁺ GC. For quantification of GC proliferation, sections were immunostained for TFAP2C, MAGEA4, and Ki-67. Proliferative GC (Ki67⁺) were quantified for TFAP2C⁺ GC in first-trimester samples and for TFAP2C⁺ GC and total GC (TFAP2C⁺ or MAGEA4⁺), on second-trimester samples, as a proportion of proliferative TFAP2C⁺ GC or GC divided by the total number of GC. Total GC proliferation was not calculated because MAGEA4⁺ GC proliferation in first-trimester fetal testes were insufficient to enable meaningful statistical analysis.

For fetal ovary analyses, sections (one per cultured piece) were co-immunostained for TFAP2C (to identify all GC) and Hoechst as a counterstain. The total number of TFAP2C⁺ GC per section was quantified and expressed relative to the total tissue section area (TFAP2C⁺ GC per micrometer squared). Proliferative TFAP2C⁺ GC (Ki67⁺) were quantified as described for fetal testes.

Gene Expression Analysis

RNA extraction was performed using the RNeasy[®] Micro Kit (Qiagen) according to manufacturer's instructions. For hanging drop cultures, all tissue pieces from each gonad were pooled. For *in vivo* rat studies, both ovaries from the same fetus were pooled, whereas a single testis from each fetus yielded sufficient cDNA for analysis. For NTERa2 cells, the RNA phase of the Trizol was separated and extracted using RNeasy[®] Micro Kit. RNA was converted into cDNA using a Vilo kit (Invitrogen) according to the manufacturer's instructions. Gene expression analysis from the cDNA was performed using the Applied Biosystems Taqman[™] RT kit (Applied Biosystems). Quantitative real time polymerase chain reaction (qRT-PCR) was performed using the ABI Prism Sequence Detection System (Applied Biosystems). Expression of epigenetic regulators in human (*TET1*, *EZH2*, *DNMT3a*, *DNMT3b*) and rat (*Tet1*, *Ezh2*, *Dnmt3a* and *Dnmt3b*), in addition to germ cell pluripotency (*POU5F1*, *TFAP2C* and *NANOG*) genes were determined using the Roche Universal Probe Library (Roche Applied Sciences) (see "NTERA2—primer sequences for gene expression analysis" in the Supplemental Material for primer sequences). The ribosomal 18S internal control (Applied Biosystems) and the transcription factor TATA-binding protein (*TBP*) (NTERa2 cell analysis only) were used as reference genes to correct the expression of the gene of interest. All samples were analyzed in duplicate. It was not possible to analyze *Dnmt3a* and *Dnmt3b* expression for *in vitro* rat gonad cultures (acetaminophen or ibuprofen) or *in vivo* rat fetal testes (e15.5 or e17.5) or ovaries (e17.5) because these experiments

were conducted prior to the assessment of *Dnmt* genes and no further tissue/mRNA was available.

H3K27me3 ELISA

Treated NTERA2 cells ($n = 3$ experiments, each in triplicate) were collected and crude histones extracted using the trimethylated lysine 27 at histone 3 (H3K27me3) ELISA kit (Active Motif) according to manufacturer's instructions. ELISA plates were pre-coated with an H3 antibody. H3K27me3 standards and the extracted crude histones from each sample were added to the appropriate well. After incubation, the primary antibody was added and incubated for 1 h. A secondary HRP conjugated antibody was then added followed by incubation for 1 h. The colorimetric reaction was initiated with a developing solution, which reacts with the HRP conjugate and produces a colorimetric signal. The reaction was stopped after 5 min by adding a stop solution to all wells. All samples were determined in duplicate. Absorbance was measured using a spectrophotometer (LabSystems; 450 nm wavelength). Known H3K27me3 standards were used to construct a standard curve, which was used to calculate total H3K27me3 per well. Total H3K27me3 per sample was expressed relative to the total protein concentration determined by the Bradford method (Thermo Fisher).

Statistics

For human fetal gonad cultures and xenograft studies, results were analyzed by two-factor ANOVA with replication. This enabled us to account for interindividual variation between fetuses as well as variation between tissue pieces or xenografts (= the replicates) from the same fetus, as previously described (Mitchell et al. 2012). For xenografts, all tissue pieces from the same fetus were treated as replicates, irrespective of which host mouse they derived from because variation between xenografts within and between hosts for the same fetus were comparable, and it was considered that this method of analysis enabled the most accurate measure of intrinsic variability (or error) in the xenograft model. Results for NTERA2 cell studies were also analyzed by two-factor ANOVA with replication, taking account of repeat experiments ($n = 3$) and replicates ($n = 3$) within each experiment. For rat *in vivo* and *in vitro* studies, analysis was performed using paired (*in vitro*) or unpaired (*in vivo*), two-tailed *t*-tests. $p < 0.05$ was used to determine significance.

Ethics Statement

Human fetal testes were obtained after elective termination of pregnancy, according to the Declaration of Helsinki–Ethical Principles for Medical Research Involving Human Subjects. Ethical approval for the study was obtained from the South East Scotland Research Ethics Committee (reference no. LREC08/S1101/1). Women gave written informed consent. Animal studies received specific approval by the UK Home Office (PPL 60/4564), including ethical approval, and were performed according to the Animal (Scientific Procedures) Act 1986.

Results

Effects of 7-d Acetaminophen or Ibuprofen Exposure on GC in Hanging Drop Cultures of First-trimester Human Fetal Gonads

Analgesic effects during human pregnancy may depend on the gestational period (Kristensen et al. 2011; Snijder et al. 2012), so we investigated effects in first and second-trimester fetal gonads. GC effects during the first trimester were investigated using

hanging drop culture (Jørgensen et al. 2015), in which testis and ovary samples were exposed to vehicle or analgesic (acetaminophen or ibuprofen; each 10 μ M) for 7 d (Figure 1A). Cultured tissue pieces appeared healthy. GC ratios were calculated using TFAP2C⁺ (gonocytes) and MAGEA4 (prespermatogonia; testis only) as GC markers, and SOX9 to mark Sertoli cells (see Figure S1A–C) in testes.

Relative to vehicle controls, acetaminophen-treated testicular tissue had significantly lower numbers of total GCs (–18%; Figure 2A,B) and gonocytes (–28%; Figure 2C,D). Ibuprofen treated tissue had significantly fewer gonocytes than controls (–22%; Figure 2C,D), but the reduction in total GCs was not significant (Figure 2A,B). MAGEA4⁺ GC numbers in first-trimester fetal testes were insufficient to enable meaningful statistical analysis (data not shown). Apoptotic cells (cleaved caspase 3⁺) were rare (see Figure S2A–C) across all treatments, so quantification was not performed. Proliferating (Ki67⁺) TFAP2C⁺ gonocytes (Figure S1D) also were significantly reduced in testis tissue exposed to acetaminophen (–22%) or ibuprofen (–28%) relative to controls (Figure 2E,F), consistent with a reduction in proliferation versus an increase in apoptosis as the underlying cause of analgesic-induced reduction in gonocyte numbers (Figure 2C,D).

In fetal human ovary cultures, numbers of TFAP2C⁺ GC were significantly reduced in tissue exposed to acetaminophen (–43%) or ibuprofen (–49%) relative to controls (Figure 3A,B). Apoptotic cells were rare across all treatments (see Figure S2D–F). Relative to controls, ovarian TFAP2C⁺ GC proliferation was significantly reduced after culture with acetaminophen (–25%) or ibuprofen (–35%) (Figure 3C,D).

Effect of 7-d Acetaminophen or Ibuprofen Exposure on GC in Second-trimester Human Fetal Testis Xenografts

Second-trimester testis tissue was xenografted into nude mice as described previously (van den Driesche et al. 2015). Treatment of host mice with acetaminophen or ibuprofen for 7 d (Figure 1B) significantly reduced total GC number (TFAP2C⁺ + MAGEA4⁺) in xenografts by 43% (Figure 4A,B) and 53% (Figure 4C,D), respectively, in comparison with vehicle-treated hosts. For acetaminophen- and ibuprofen-exposed samples, this reduction appeared to be mainly due to a reduction in TFAP2C⁺ GC, [for acetaminophen-exposed tissue, –32% (Figure 4E,F); for ibuprofen-exposed tissue, –60% (Figure 4G,H)], although this reduction was significant only for acetaminophen. MAGEA4⁺ GC number was also reduced following 7 d of acetaminophen or ibuprofen exposure, though not significantly [–50% (see Figure S3A,B); and –43% (see Figure S3C,D), respectively]. Proliferation of total GC was reduced after 7 d of acetaminophen (–40%; Figure 5A,B) and ibuprofen exposure (–21%; Figure 5C,D), but this was not significant. Similarly, proliferation of TFAP2C⁺ GC was reduced after 7 d of acetaminophen or ibuprofen exposure, but not significantly [–34% (Figure 5E,F); and –20% (Figure 5G,H), respectively].

Effect of 1-d Acetaminophen Exposure on GC in Second-trimester Human Fetal Testis Xenografts

As acetaminophen use in pregnancy may be mainly for short periods (≤ 24 h), we investigated whether 1-d exposure to acetaminophen, followed by 6 d of vehicle treatment altered GC number (Figure 1B). This exposure led to a significant reduction in total GC (22%; Figure 6A,B) and TFAP2C⁺ GC number (17%; Figure 6C,D) in xenografts, although the reduction was less pronounced compared with that seen after 7-d acetaminophen exposure (Figure 4A,B and E,F). MAGEA4⁺ GC number was not significantly altered by 1-d acetaminophen exposure (see Figure S3E–F). GC proliferation (total or TFAP2C⁺) was

1st trimester fetal testis culture

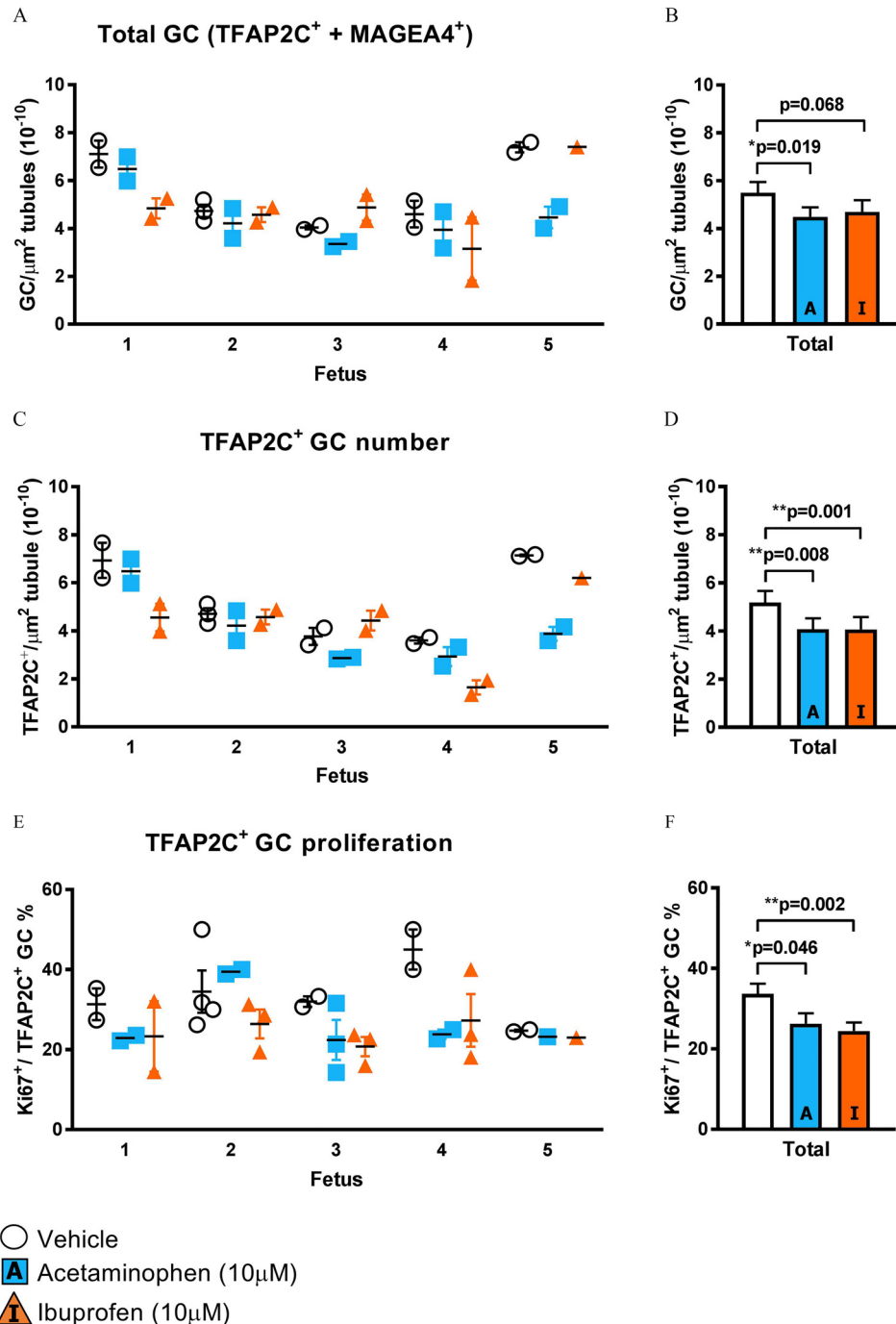


Figure 2. Effect of analgesic exposure of first-trimester fetal human testis tissue for 7 d in hanging drop culture on germ cell (GC) number. Testis tissue pieces (~1 mm³) from five fetuses (8–11 GW) were cultured for 7 d with vehicle, acetaminophen (A; 10 μM) or ibuprofen (I; 10 μM) (1–4 pieces from each fetus per treatment). Tissue was immunostained to distinguish Sertoli cells (SOX9⁺), undifferentiated GC (TFAP2C⁺) and differentiated GC (MAGEA4⁺), and GC counts were determined within a 1-μm² field of SOX9⁺ tubule cells. Proliferating GC were identified by additional staining for Ki67, and the percentage of proliferating TFAP2C⁺ cells was determined by dividing all proliferative TFAP2C⁺ by the total number of TFAP2C⁺. (A) Individual data points represent total GC counts (TFAP2C⁺ + MAGEA4⁺ cells) for each 1-μm² tissue piece per treatment and fetus, with horizontal lines indicating the mean for each treatment and fetus. (B) Mean (± SEM) total GC counts over all tissue pieces and fetuses for each treatment. (C) Individual data points represent TFAP2C⁺ GC counts for each tissue piece per treatment and fetus, with horizontal lines indicating the mean for each treatment and fetus. (D) Mean (± SEM) TFAP2C⁺ GC counts over all tissue pieces and fetuses for each treatment. (E) Individual data points represent the percentage of proliferating Ki67⁺ TFAP2C⁺ GC in each tissue piece per treatment and fetus, with horizontal lines indicating the mean for each treatment and fetus. (F) Mean (± SEM) percentage of Ki67⁺ TFAP2C⁺ cells over all tissue pieces and fetuses for each treatment. *p*-Values shown in B, D, and E compare values in treated samples to vehicle controls using two-factor ANOVA to account for tissue replicates for each fetus and multiple fetuses per treatment. Fetuses 1–5 were 8, 10, 11, 9, and 8 GW of age, respectively. Note: GW, gestational weeks; Ki67, cellular marker for proliferation; SEM, standard error of the mean.

1st trimester fetal ovary culture

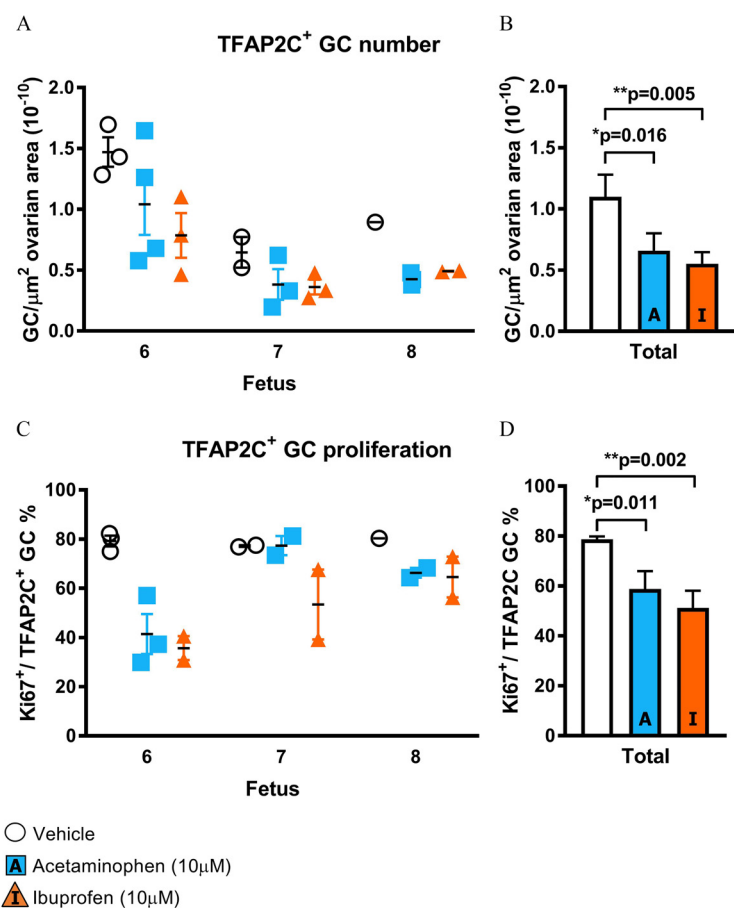


Figure 3. Effect of analgesic exposure of first-trimester fetal human ovary tissue for 7 d in hanging drop culture on germ cell (GC) number. Ovary tissue pieces (~1 mm³) from three fetuses (9–11 GW) were cultured for 7 d with either vehicle, acetaminophen (A; 10 μM) or ibuprofen (I; 10 μM). Tissue was fixed and double-immunostained for TFAP2C and Hoechst to count the number of undifferentiated TFAP2C⁺ GC per 1-μm² field of ovary tissue. In addition, cells were stained for Ki67 to determine the percentage of proliferating TFAP2C⁺ cells, which was determined by dividing all proliferative TFAP2C⁺ by the total number of TFAP2C⁺. (A) Individual data points represent TFAP2C⁺ GC counts for each individual tissue piece per treatment and fetus, with horizontal lines indicating the mean for each treatment and fetus. (B) Mean (±SEM) TFAP2C⁺ GC counts over all tissue pieces and fetuses for each treatment. (C) Individual data points represent the percentage of proliferating Ki67⁺ TFAP2C⁺ GC for each tissue piece per treatment and fetus, with horizontal lines indicating the mean for each treatment and fetus. (D) Mean (±SEM) percentage of Ki67⁺ TFAP2C⁺ cells over all tissue pieces and fetuses for each treatment. *p*-Values shown in B, D, and E compare values in treated samples with vehicle controls using two-factor ANOVA to account replicate tissue pieces for each fetus and multiple fetuses per treatment. Fetuses 6–8 were 9, 11, and 9 GW, respectively. Note: GW, gestational weeks; Ki67, cellular marker for proliferation; SEM, standard error of the mean.

reduced after 1-d acetaminophen exposure, but not significantly (–34%, and –34%, respectively; Figure 6E–H).

Effects of Acetaminophen, Ibuprofen, and EP2 + EP4 Antagonists on NTera2 Cell Number

In view of the effects of analgesic exposure on GC number in human fetal testis tissue, we utilized NTera2 cells as a more tractable system to dissect possible mechanisms of analgesic action. In addition to investigating effects of acetaminophen and ibuprofen on NTera2 cells, we also investigated whether blockade of PGE₂ action via combined exposure to an EP2 (PF-04418948, 10 μM), and an EP4 (L-161,982, 10 μM,) receptor antagonist, induced effects similar to those of analgesics. NTera2 cells were exposed to acetaminophen (10 or 50 μM), ibuprofen (10 μM) or EP2 + EP4 antagonists (10 μM each; Figure 1C). All three exposures significantly reduced total NTera2 cell number (including both dead and viable cells) after 48 h, relative to controls (–19% for 10-μM acetaminophen, –58% for 50-μM acetaminophen,

–17% for ibuprofen, and –27% for EP2 + EP4 antagonists; Figure 7A–C) suggesting that the analgesic-induced decrease in NTera2 cell number could involve inhibition of the PGE₂ pathway. The percentage of dead NTera2 cells was significantly higher than in vehicle controls following exposure to 10-μM acetaminophen (2.97% vs. 2.13%) and to EP2 + EP4 antagonists (2.69% vs. 2.16%), but the difference was more pronounced following treatment with 50-μM acetaminophen (7.42% vs. 2.13%) (see Figure S4). Thus, we considered 50-μM acetaminophen to be a toxic dose.

To test whether the acetaminophen-induced decrease in NTera2 cell number was due to inhibition of the PGE₂ pathway, NTera2 cells were cultured either with acetaminophen (10 μM), with EP2 + EP4 agonists (Butaprost 10 μM + CAY10598 10nM) or with the two treatments combined (Figure 1C). Exposure to the EP2 + EP4 agonists alone slightly reduced NTera2 cell number, but not significantly so (–3.3%; Figure 7D), whereas cell number was significantly reduced by exposure to acetaminophen (–21%; Figure 7D), consistent with previous findings

2nd trimester fetal testis xenografts

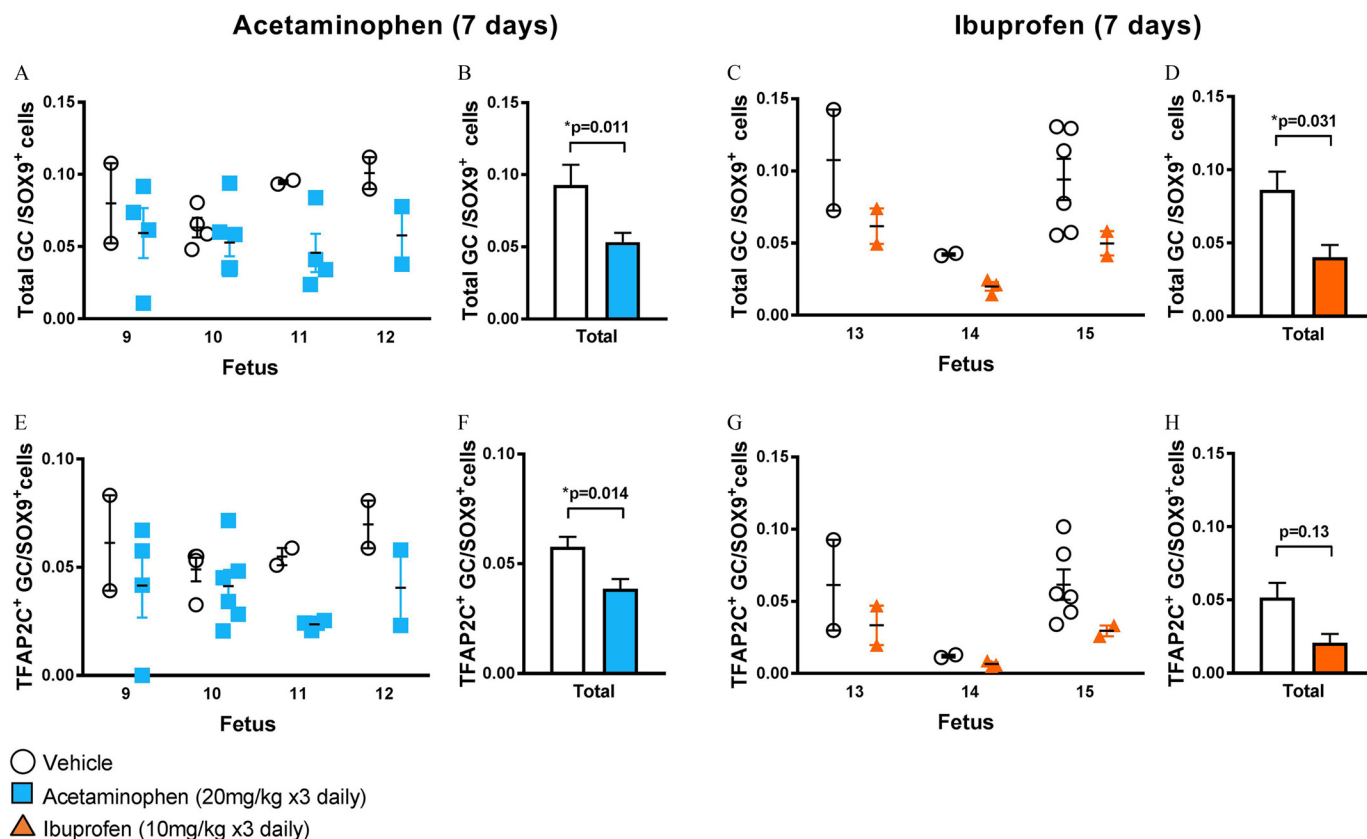


Figure 4. Effect of 7 d acetaminophen or ibuprofen exposure of second-trimester fetal human testis tissue xenografts on germ cell (GC) number. Testis tissue pieces ($\sim 1 \text{ mm}^3$) from four fetuses (acetaminophen) or three fetuses (ibuprofen) were xenografted into nude mice. Host mice were administered vehicle, acetaminophen (20 mg/kg, three times daily) or ibuprofen (10 mg/kg, three times daily) for 7 d before xenograft recovery. Tissues were fixed and immunostained to identify Sertoli cells (SOX9⁺), undifferentiated GC (TFAP2C⁺) and differentiated GC (MAGEA4⁺). Total GC counts (TFAP2C⁺ + MAGEA4⁺ cells) and TFAP2C⁺ GC counts were determined relative to the number of SOX9⁺ Sertoli cells. (A and C) Individual data points representing total GC counts (TFAP2C⁺ + MAGEA4⁺ cells) for each 1- μm^2 tissue piece per treatment and fetus, with horizontal lines indicating the mean for each treatment and fetus. (E and G) Individual data points representing TFAP2C⁺ GC counts for each tissue piece per treatment and fetus, with horizontal lines indicating the mean for each treatment and fetus. (B, D, F, and H) Means \pm SEMs over all tissue grafts for each treatment and fetus. *p*-Values shown in B, D, F, and H compare values in treated samples with vehicle controls using two-factor ANOVA to account replicate tissue pieces for each fetus and multiple fetuses per treatment. Corresponding MAGEA4⁺ GC counts after 7-d treatments with acetaminophen or ibuprofen are shown in Figure S3A–D. Fetuses 9–15 were 14, 14, 14, 17, 14, 17, and 16 GW respectively. Note: GW, gestational weeks; Ki67, cellular marker for proliferation; SEM, standard error of the mean.

(Figure 7A). In contrast, the NTera2 cell count was not significantly different from controls following coculture with 10- μM acetaminophen and the EP2 + EP4 agonists, indicating that acetaminophen-induced NTera2 cell loss requires an intact PGE signaling pathway (Figure 7D).

Effects of Acetaminophen, Ibuprofen, or EP2 + EP4 Antagonists on Expression of GC Differentiation Markers in NTera2 Cells

We previously showed that expression of the gonocyte pluripotency marker POU class 5 homeobox 1 (POU5F1 or OCT4) ended earlier in the fetal testes of rats whose dams were exposed to 350 mg/kg per day acetaminophen or 0.8 mg/kg per day indomethacin than in fetal testes from the offspring of unexposed dams (Dean et al. 2016). We therefore investigated whether expression of the established GC pluripotency markers *POU5F1*, *TFAP2C*, and *NANOG* (Rajpert-De Meyts 2006) differed from vehicle controls in NTera2 cells cultured with acetaminophen, ibuprofen, or EP2 + EP4 antagonists (10 μM each). Compared with vehicle controls, expression of *POU5F1* and *TFAP2C* was significantly lower in NTera2 cells following acetaminophen

exposure (Figure 8A), whereas only *POU5F1* expression was significantly lower following ibuprofen exposure (Figure 8B). Expression of all three genes was significantly lower than vehicle controls in NTera2 cells cultured with EP2 + EP4 antagonists (Figure 8C).

Effects of Acetaminophen, Ibuprofen, or EP2 + EP4 Antagonists on Expression of Epigenetic Regulatory Genes in NTera2 Cells

Previous studies showed intergenerational consequences of *in utero* acetaminophen/NSAID exposure in rats (Dean et al. 2016) and other publications demonstrate that PGE₂ may fundamentally regulate the epigenetic machinery (Arosh et al. 2015; Venza et al. 2012). Therefore, we hypothesized that exposure to acetaminophen, ibuprofen, or EP2 + EP4 antagonists might exert similar effects on epigenetic regulatory genes in NTera2 cells.

Expression of the DNA methyltransferases *DNMT3a* and *DNMT3b* in NTera2 cells was decreased relative to vehicle controls following exposure to acetaminophen, ibuprofen, and the EP2 + EP4 antagonists, though the difference in *DNMT3a*

2nd trimester fetal testis xenografts

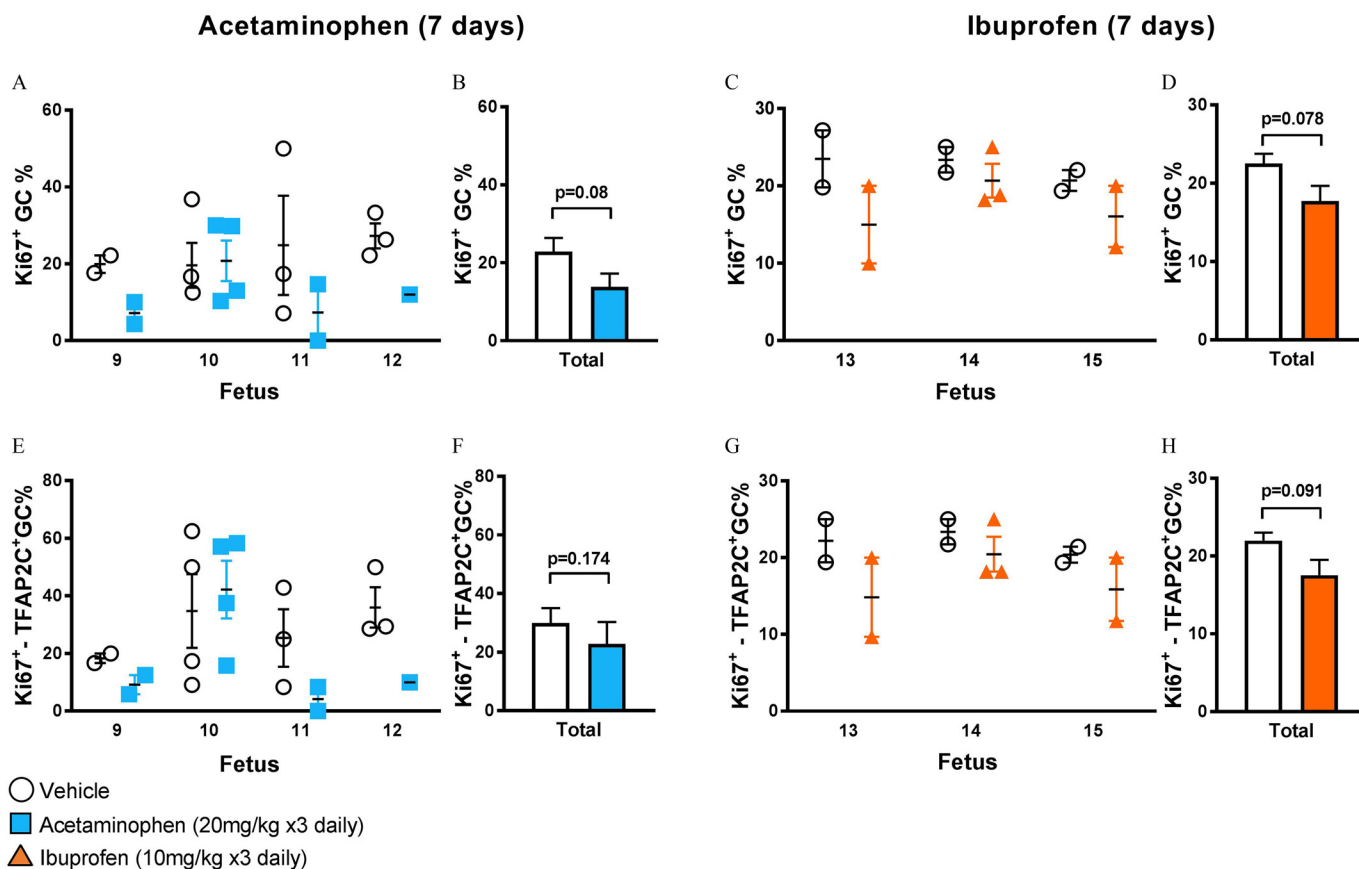


Figure 5. Effect of 7-d acetaminophen or ibuprofen exposure of second-trimester fetal human testis tissue xenografts on germ cell (GC) proliferation. Testis tissue pieces ($\sim 1 \text{ mm}^3$) from four fetuses (acetaminophen) or three fetuses (ibuprofen) were xenografted into nude mice. Host mice were administered vehicle, acetaminophen (20 mg/kg, three times daily) or ibuprofen (10 mg/kg, three times daily) for 7 d before xenograft recovery. Tissues were fixed and immunostained to identify undifferentiated GC (TFAP2C⁺) and differentiated GC (MAGEA4⁺), with Ki67 staining to identify proliferating cells. Percentage of proliferating TFAP2C⁺ cells was determined by dividing all proliferative TFAP2C⁺ by the total number of TFAP2C⁺. (A and C) Individual data points representing the percentage of proliferating Ki67⁺ GC for each tissue piece per treatment and fetus, with horizontal lines indicating the mean for each treatment and fetus. (E and G) Individual data points representing the percentage of proliferating Ki67⁺ TFAP2C⁺ GC for each tissue piece per treatment and fetus, with horizontal lines indicating the mean for each treatment and fetus. (B, D, F, and H) Mean (\pm SEM) percentage of Ki67⁺ GC or TFAP2C⁺ cells over all tissue pieces and fetuses for each treatment. *p*-Values shown in B, D, F, and H compare values in treated samples with vehicle controls using two-factor ANOVA to account for replicate tissue pieces for each fetus and multiple fetuses per treatment. Fetuses 9–15 were 14, 14, 14, 17, 14, 17, and 16 GW, respectively. Note: GW, gestational weeks; Ki67, cellular marker for proliferation; SEM, standard error of the mean.

expression was significant for ibuprofen only, whereas the difference in *DNMT3b* expression was significant for acetaminophen and EP2 + EP4 antagonist exposures only (Figure 8D–F). Expression of *TET1*, which plays a role in DNA and histone methylation, was significantly increased compared with controls after all three treatments, whereas expression of *EZH2*, the enzymatic component of the PRC2 complex responsible for H3K27me3 histone methylation, was increased relative to controls following exposure to ibuprofen (25% increase) and EP2 + EP4 antagonists, (31% increase) though the difference was significant only for the latter (Figure 8D–F). However, the relative amount of H3K27me3, as measured by ELISA, was significantly higher than in vehicle controls after culture of NTERA2 cells with acetaminophen, ibuprofen, or EP2 + EP4 antagonists (Figure 8G).

In vitro and in vivo Effects of Acetaminophen, Ibuprofen, or EP2 + EP4 Antagonists on Fetal Rat Gonads

Findings from human fetal gonad cultures and xenografts, and *in vitro* studies of the NTERA2 human GC tumor-derived cell line,

suggest potential effects of analgesic exposures during pregnancy that may be mediated by PGE₂. We next evaluated the effects of analgesic exposures and PGE₂ antagonists on epigenetic regulatory genes using fetal rat ovarian or testicular tissue in hanging drop cultures, to determine whether findings were consistent between human and rat tissues, before proceeding with *in vivo* studies using a rat model. Tissue pieces were cultured for 48 h with or without exposure to acetaminophen (10 μM), ibuprofen (10 μM), or EP2 + EP4 antagonists (10 μM L-161,982 + 10 μM PF04418948) (Figure 1D). As for NTERA2 cells (Figure 8D–F), *Tet1* expression in fetal (e15.5) rat testes and ovaries was consistently increased relative to controls after exposure to any of the three treatments, although in the case of fetal ovaries, the ibuprofen-induced increase did not reach statistical significance (Figure 9A–F). *Ezh2* expression was higher in testis and ovaries after exposure to ibuprofen, as in NTERA2 cells, but, in contrast with NTERA2 cells, *Ezh2* expression was also increased following exposure to acetaminophen, and was lower than in controls following culture with EP2 + EP4 antagonists (significant for ovary cultures). Expression of *Dnmt3b* was significantly lower than

2nd trimester fetal testis xenografts

Acetaminophen (1 day)

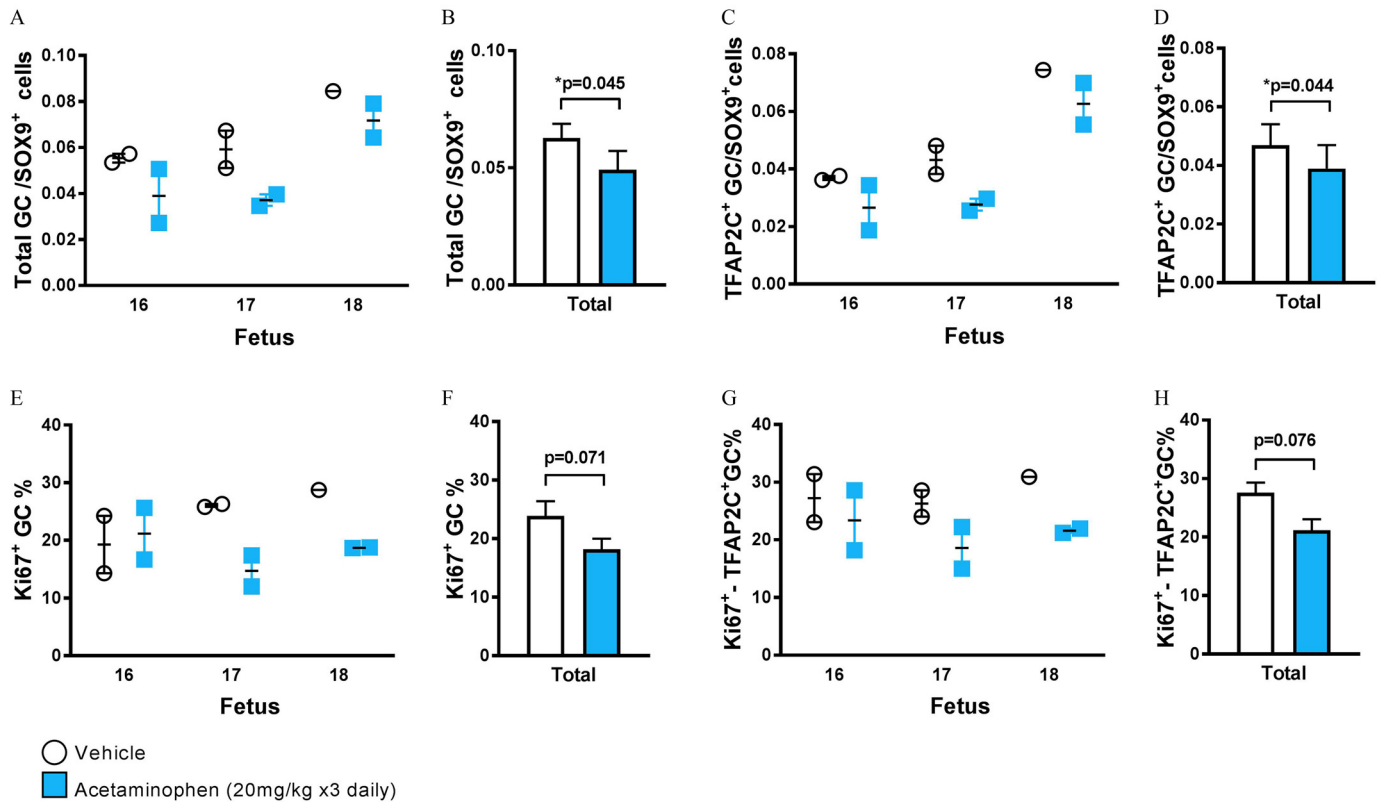


Figure 6. Effect of 1-d acetaminophen exposure of second-trimester fetal human testis tissue xenografts on germ cell (GC) number and GC proliferation. Testis tissue pieces (~1 mm³) from three fetuses were xenografted into nude mice. Host mice were administered vehicle or acetaminophen (20 mg/kg, three times daily) for 1 d and xenografts were retrieved 6 d later. Tissues were fixed and immunostained to identify Sertoli cells (SOX9⁺), undifferentiated GC cells (TFAP2C⁺), and differentiated GC (MAGEA4⁺), with Ki67 staining to identify proliferating cells. Total GC counts (TFAP2C⁺ + MAGEA4⁺ cells) and TFAP2C⁺ GC counts were determined relative to the number of SOX9⁺ Sertoli cells. Percentage of proliferating TFAP2C⁺ cells was determined by dividing all proliferative TFAP2C⁺ by the total number of TFAP2C⁺. (A) Individual data points represent total GC counts (TFAP2C⁺ + MAGEA4⁺ cells) for each tissue piece per treatment and fetus, with horizontal lines indicating the mean for each treatment and fetus. (B) Mean (± SEM) total GC counts over all tissue pieces and fetuses for each treatment. (C) Individual data points represent TFAP2C⁺ GC counts for each tissue piece per treatment and fetus, with horizontal lines indicating the mean for each treatment and fetus. (D) Mean (± SEM) TFAP2C⁺ GC counts over all tissue pieces and fetuses for each treatment. (E) Individual data points represent the percentage of proliferating Ki67⁺ GC in each tissue piece per treatment and fetus, with horizontal lines indicating the mean for each treatment and fetus. (F) Mean (± SEM) percentage of Ki67⁺ GC. (G) Individual data points represent the percentage of proliferating Ki67⁺ TFAP2C⁺ GC in each tissue piece per treatment and fetus, with horizontal lines indicating the mean for each treatment and fetus. (H) Mean (± SEM) percentage of Ki67⁺ TFAP2C⁺ cells. p -Values shown in B, D, F, and H compare values in treated samples with vehicle controls using two-factor ANOVA to account for replicate tissue pieces for each fetus and multiple fetuses per treatment. Fetuses 16–18 were 15, 19, and 14 GW, respectively. Note: GW, gestational weeks; Ki67, cellular marker for proliferation; SEM, standard error of the mean.

controls after exposure to EP2+EP4 antagonists in both testis and ovary cultures (Figure 9C,F), whereas expression of *Dnmt3a* was significantly lower in ovary cultures but slightly higher in testis cultures, but not significantly so (Figure 8C,F). *Dnmt3a* and *Dnmt3b* expression were not evaluated in rat testis or ovary cultures exposed to acetaminophen or ibuprofen.

To provide *in vivo* evidence for the relevance of the identified gene expression changes induced by analgesics *in vitro*, we used acetaminophen exposure because this analgesic was more frequently associated with significant effects in the human fetal testis xenografts. Pregnant rats received 350 mg/kg acetaminophen (equivalent to human dose of ~60 mg/kg based on calculation of body surface area; Reagan-Shaw et al. 2008) by gavage once daily from e13.5 until the day before sampling (either e15.5 or e17.5) (Figure 1E). Similar to the *in vitro* culture studies, *Tet1* expression was increased relative to controls in both the fetal testis (e15.5 and e17.5; Figure 10A,B) and ovary (significant for e15.5 only; Figure

10C,D). *Ezh2* expression was significantly higher than in controls in e17.5 testes (Figure 10B) and e15.5 ovaries (Figure 10C) after acetaminophen exposure. Expression of *Dnmt3a* was significantly lower, whereas expression of *Dnmt3b* was only slightly, but not significantly, than controls in e15.5 ovaries following *in-vivo* acetaminophen exposure (Figure 10C), whereas for *in vitro* ovary cultures, the acetaminophen-induced reduction in *Dnmt3A* and *Dnmt3B* expression was significant for both genes (Figure 9F). *Dnmt3a* and *Dnmt3b* expression were not evaluated in testis (e15.5 or e17.5) or ovary cultures (e17.5) exposed to acetaminophen or ibuprofen.

Discussion

Our results show that, under our experimental conditions in our model systems, exposure to the analgesics acetaminophen and ibuprofen, at human therapeutically relevant levels, causes significant

NTera2 cell culture

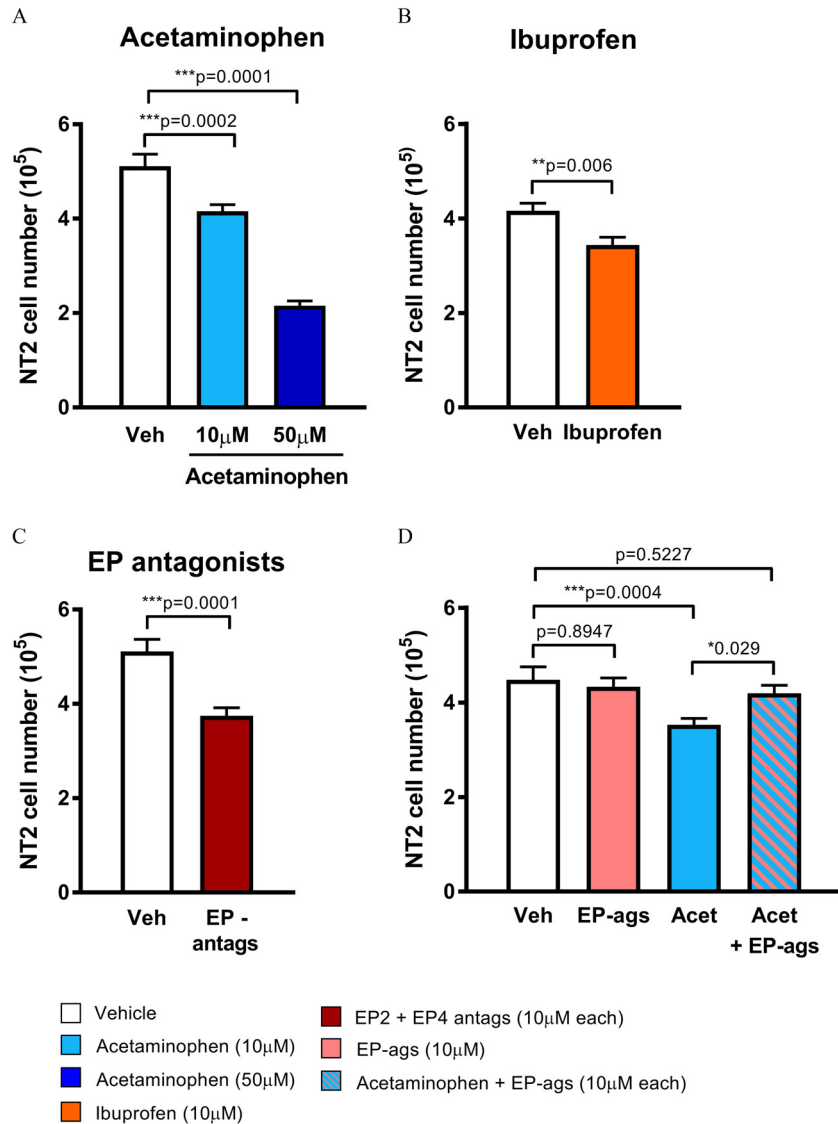


Figure 7. Effect of exposure of NTera2 cells to analgesics or prostaglandin E₂-receptor modulators on cell number. NTera2 cells (2×10^5 ; $n = 3$ for A, B and C, $n = 4$ for D) were cultured for 48 h in medium supplemented with either vehicle (Veh), (A) acetaminophen (10–50 μM), (B) ibuprofen (10 μM), or (C) PGE₂ antagonists [EP-antags: 10 μM L-161,982 (EP2 antagonist) + 10-μM PF04418948 (EP4 antagonist)]. (D) NTera2 cells were cultured with PGE₂ EP2 + EP4 receptor agonists [EP-ags: 10-μM Butaprost (EP2 agonist) + 10-nM CAY10598 (EP4 agonist)] for 4 h before media were changed to EP2 + EP4 receptor agonists only, acetaminophen (10 μM) only, or acetaminophen + EP2/EP4 agonists (Acet + EP-ags) and cultured for another 44 h. Values shown are mean \pm SEM numbers of NTera2 (including both, dead and viable cells) from three independent experiments with three replicates for A, B, and C, and four replicates for D. *p*-Values compare values in treated samples with vehicle controls using two-factor ANOVA. Corresponding data for the percentage of dead NTera2 cells following each treatment are shown in Figure S4. Note: ag, agonist; antag, antagonist; SEM, standard error of the mean.

reductions in GC number in both human fetal testes and ovaries, as well as altering expression of GC differentiation markers and epigenetic regulatory genes in NTera2 cells, a surrogate for fetal GC. The analgesic-induced reduction in GC (total and TFAP2C⁺) number in the human fetal testis was evident for samples from both first trimester (in culture) and second trimester (xenografts), and in the latter could be induced even with a single day's treatment with acetaminophen using a human-relevant therapeutic dose/regimen. Because studies in the United States (Werler et al. 2005), Germany (Bremer et al. 2017), France (Philippat et al. 2011), Denmark (Jensen et al. 2010), Norway (Harris et al. 2017), and Sweden (Wolgast et al. 2017) and a multi-national study (Europe, North/South America, and Australia) (Lupattelli et al. 2014) have documented that many

pregnant women may take analgesics (mainly acetaminophen), the present findings raise the possibility of fetal germ cell effects in human pregnancy. This is reinforced by previous studies that have shown that analgesic exposure of pregnant rodents can affect the future fertility of newborn females (Dean et al. 2016; Holm et al. 2016). Our results suggest that the analgesic effects on fetal GC may be mediated through the PGE₂ pathway, and may affect the expression of key epigenetic regulatory genes. This raises the possibility that analgesic exposure might alter the epigenome of fetal GC, potentially affecting the next generation, as previously demonstrated in the rat (Dean et al. 2016).

Because direct translation of our earlier findings of adverse effects of analgesic exposure on fetal GC in the rat (Dean et al. 2016) to the human is not possible, we employed *in vitro* and *ex*

Ntera2 cell culture

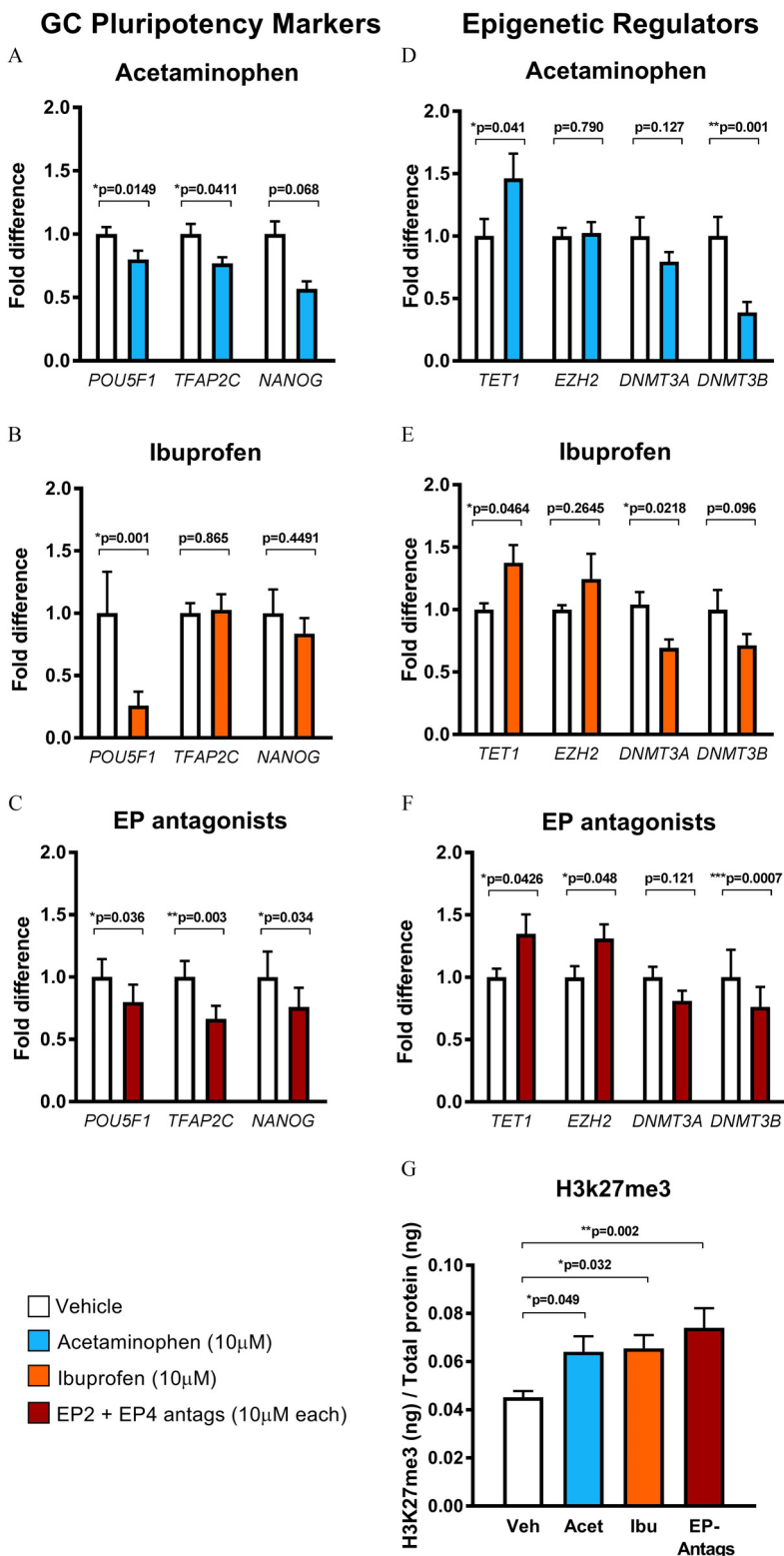


Figure 8. Effect of exposure of Ntera2 cells to analgesics or prostaglandin E_2 antagonists on mRNA expression of germ cell (GC) pluripotency markers, epigenetic regulatory genes, and overall H3K27me3 levels. Ntera2 cells (2×10^5 ; $n = 3$) were cultured for 48 h in medium supplemented with vehicle (Veh), acetaminophen (Acet; 10–50 μ M), ibuprofen (Ibu; 10 μ M) or EP2 + EP4 antagonists [EP-antags (10- μ M L-161,982; EP2 antagonist +10- μ M PF04418948; EP4 antagonist)]. Results show mRNA expression relative to the vehicle complementary DNA (open bars) for the GC pluripotency markers *POU5F1*, *TFAP2C*, and *NANOG* (A, C, and E, respectively) and for the epigenetic regulatory genes *TET1*, *EZH2*, *DNMT3a*, and *DNMT3b* (B, D, and F, respectively) after exposure of Ntera2 cells to acetaminophen (blue), ibuprofen (orange), or EP2 + EP4 antagonists (maroon) ($n = 3$). (G) Relative amount of H3K273 present in the Ntera2 cells after culture with the different treatments. Graphs show mean \pm SEM from three independent experiments with three replicates in each experiment and statistical analysis was by two-factor ANOVA. Note: antagon, antagonist; SEM, standard error of the mean.

In-vitro rat gonad culture

Testis

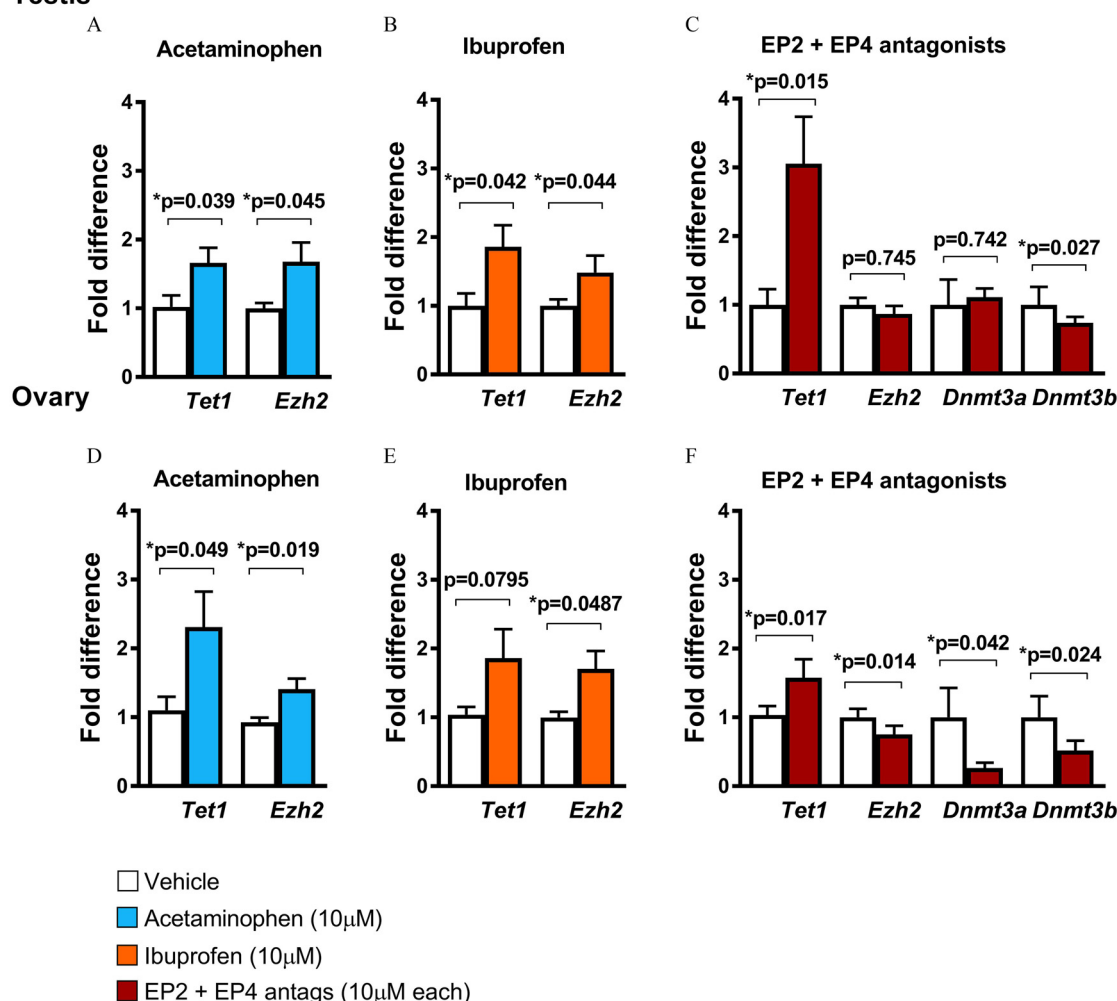


Figure 9. Effect of exposure of rat fetal gonads to analgesics or prostaglandin E_2 antagonists *in vitro* on mRNA expression of epigenetic regulatory genes. Testis and ovary tissue pieces ($\sim 1 \text{ mm}^3$) from e15.5 fetal rats were cultured for 48 h with either vehicle (open bars), acetaminophen (blue; 10 μM), ibuprofen (orange; 10 μM), or EP2 + EP4 antagonists [maroon; 10- μM L-161,982 (EP2 antagonist) + 10- μM PF04418948 (EP4 antagonist)]. (A–F) mRNA expression relative to the vehicle complementary DNA (open bars) for *Tet1*, *Ezh2*, *Dnmt3a*, and *Dnmt3b* for cultured fetal testes (A–C) or ovaries (D–F) (mean \pm SEM, $n = 13$ –30). Data were analyzed by paired *t*-test. Note: antag, antagonist.

vivo (xenograft) model systems using fetal testicular or ovarian tissue or Ntera2 cells. Exposure to either acetaminophen or ibuprofen at human-relevant concentrations reduced GC/Ntera2 cell number in all of these systems, consistent with previous studies showing decreased GC number after analgesic exposure during pregnancy in rodents (Dean et al. 2016; Holm et al. 2016). In the human fetal testis, in culture (first trimester) the effect on GC number resulted from a reduction in the TFAP2C⁺ gonocyte population, whereas in xenografts (second trimester) the reduction in germ cell number appeared to involve TFAP2C⁺ and more differentiated prespermatogonia (MAGEA4⁺). TFAP2C⁺ GC in the human fetal testis are highly proliferative (Mitchell et al. 2010), and our results tentatively suggest that analgesics most likely induce GC loss via reduced proliferation. In fetal human ovary cultures, the highly proliferative premeiotic oogonia (TFAP2C⁺) were reduced in number following analgesic exposure, similar to findings in the fetal testis. Analgesic targeting of TFAP2C⁺ GC may be related to the probable mechanism of analgesic action via PGE₂, as discussed below, given that the PGE₂ pathway is important in modulating proliferation of (pluripotent)

stem cells in various other systems (Hoggatt et al. 2009; Wang et al. 2013; Xia et al. 2012; Yun et al. 2009; Wu et al. 2017; Ho et al. 2017).

In the xenograft model, which recapitulates normal human fetal testis GC development (Mitchell et al. 2010), TFAP2C⁺ GC number was significantly reduced by a single day's exposure to acetaminophen using a human-relevant exposure regimen. Similarly, in human fetal gonad cultures, analgesic-induced reduction in GC number occurred at concentrations that are achieved in blood after normal human usage of acetaminophen or ibuprofen (Albert et al. 2013; Janssen and Venema 1985; Mazaud-Guittot et al. 2013). The fact that analgesic exposure consistently induced GC loss in the human fetal testis and ovary in each model system used, as well as causing similar GC loss in rats in fetal gonads after analgesic exposure *in vivo* (Dean et al. 2016), demonstrates that this is a consistent effect. This raises concerns about potential consequences of analgesic use in human pregnancy and supports the need for further investigation. In rats, fetal GC reduction in males can be compensated postnatally as the differentiated GC resume proliferation after birth (Dean et al. 2016), and it is likely that this would also occur

In-vivo exposure of rat fetal gonads

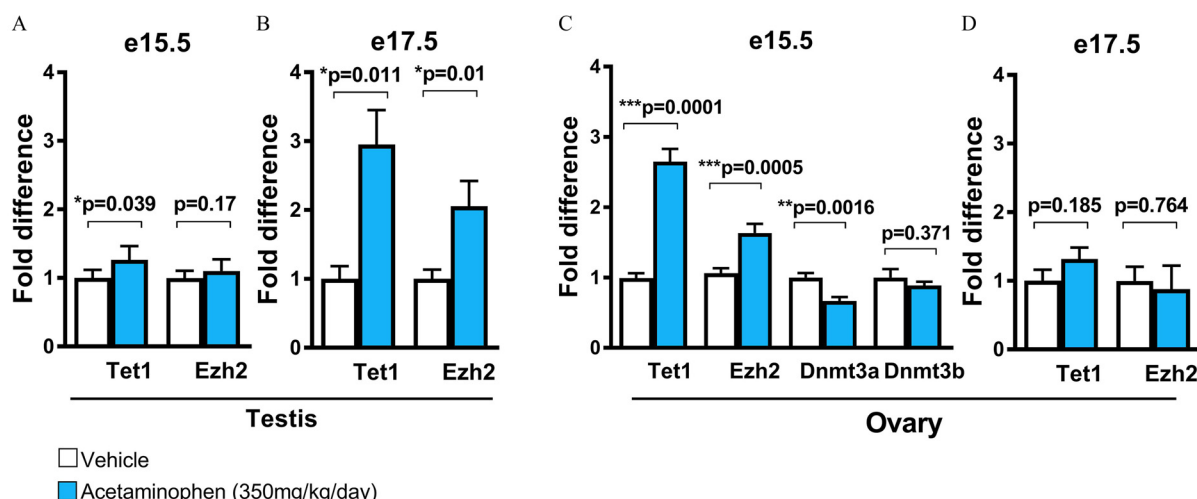


Figure 10. Effect of exposure of pregnant rats to acetaminophen on mRNA expression of epigenetic regulatory genes in fetal testes and ovaries. Pregnant rats were administered acetaminophen (350 mg/kg once daily), commencing on e13.5, and fetal gonadal tissue was collected on e15.5 or e17.5, 3 h after the final maternal treatment. (A–D) mRNA expression relative to the vehicle cDNA for the same genes after acetaminophen exposure *in vivo* for e15.5 (A) and e17.5 (B) testes and for e15.5 (C) and e17.5 (D) ovaries (mean \pm SEM n = 7–25). Data were analyzed by two-tailed, unpaired *t*-test.

in human males. However, proliferation of GC in the human ovary is restricted by subsequent entry to meiosis from 9 wk gestation (Bendsen et al. 2006; Gondos et al. 1986), so that any GC loss after this period could have long-term consequences for oocyte reserves and reproductive lifespan (Nelson et al. 2013). In keeping with this, previous studies in pregnant rats showed that analgesic-induced decrease in fetal GC number translated into a decrease in adult ovarian weight and reduced fertility in adulthood (Dean et al. 2016). This might be investigated in humans by relating analgesic use in pregnancy to serum anti-Müllerian hormone (AMH) concentrations in female offspring, given that AMH levels may indicate oocyte reserves under certain conditions (Dewailly et al. 2014).

A key aim of the present study was to investigate a potential mechanism for the effects of acetaminophen and ibuprofen on fetal GC. Both analgesics have established effects on prostaglandin pathways (Aminoshariae and Khan 2015; Anderson 2008; Van Hecken et al. 2000). Our *in vivo* rat studies have shown that analgesic exposure reduces PGE₂ levels in fetal gonads (Dean et al. 2016) and fetal GC are PGE₂ targets in both rats (Dean et al. 2016) and humans (Bayne et al. 2009), so we focused on this pathway. Because isolation and culture of human fetal GC was not feasible, we used NTera2 cells. These cells derive from a human pluripotent embryonal carcinoma cell line that expresses markers (e.g., TFAP2C, POU5F1, NANOG) similar to pluripotent human fetal GCs (Knower et al. 2007). Our results show that, in our model systems, analgesic effects on NTera2 cell number and pluripotency gene expression are mimicked by culturing with EP2 + EP4 receptor antagonists (which block PGE₂ action). Moreover, the acetaminophen-induced decrease in NTera2 cell number was prevented by concomitant exposure to EP2 + EP4 agonists. Thus analgesic effects on NTera2 cells may be mediated via the PGE₂ pathway. This is supported by our finding that PGE₂ antagonism induced a number of gene expression changes that were comparable to those induced by acetaminophen, and to a lesser extent ibuprofen, in both human (NTera2 cells) and rat (fetal gonad) systems.

Our studies with NTera2 cells show that acetaminophen exposure or PGE₂ antagonism reduces expression of one or more GC pluripotency genes (*POU5F1*, *TFAP2C*, *NANOG*). This is consistent with previous evidence that EP2 antagonism down-

regulates pluripotency genes (*Pou5f1*, *Nanog*) in mouse embryonic stem cells (Yun et al. 2012), and abundant evidence that PGE₂ regulates proliferation and differentiation status in numerous stem cell and cancer cell types *in vitro* and *in vivo* (Jones et al. 2010; Wang et al. 2013; Xia et al. 2012; Wang et al. 2015; Wang and Dubois 2006). Exposure of human fetal testis explants to ibuprofen was also reported to reduce expression of specific germ cell genes involved in pluripotency (*POU5F1*, *LIN28A*) (Ben Maamar et al. 2017), a finding echoed presently for *POU5F1* in NTera2 cells. In human males, arrested differentiation of fetal GC has been proposed as the primary pathway to development of testicular GC cancer (TGCC), the most common malignancy among young men 20–40 y of age (Rajpert-De Meyts 2006; Mitchell et al. 2014). However, analgesic-induced reduction in expression of pluripotency genes in all of our studies (this study and Dean et al. 2016), is opposite to what is thought to underlie the origin of TGCC.

Our present results demonstrate that analgesic exposure variably induced changes in expression of a number of epigenetic regulatory genes in NTera2 cells as well as in fetal ovaries and testes from the rat (*in vitro* and *in vivo*). Although these gene expression changes were not completely consistent, they raise the possibility that the epigenome of exposed fetal GC could be altered by analgesic exposure, with unknown consequences. This would be consistent with our previous finding that fetal exposure of rats to analgesics results in intergenerational transmission of reproductive effects, including via the paternal line, which is indicative of an epigenetic mechanism (Dean et al. 2016). Our present demonstration that total H3K27me3 was significantly increased in NTera2 cells by exposure to acetaminophen, ibuprofen, or EP2/EP4 antagonists, when compared with vehicle control, raises the possibility that alterations in this repressive histone mark in specific genes could have occurred. H3K27me3 is highly expressed and dynamically regulated in fetal GC and is considered important in fetal GC differentiation (Sasaki and Matsui 2008; Seki et al. 2007). We also showed that analgesics and/or PGE₂ antagonism decreased expression of one or other of the key methyltransferase enzymes DNMT3a and DNMT3b in NTera2 cell culture and of the corresponding genes in *in vitro* rat culture (testis and ovary) and in rat *in vivo* (investigated in ovary only), which

might fit with previous studies linking PGE₂ action with DNA methylation (Huang et al. 2012; Venza et al. 2012; Xia et al. 2012). As fetal GC undergo extensive de- and remethylation during perinatal development (Guo et al. 2014; Sasaki and Matsui 2008), analgesic exposure could potentially impact this process via effects on DNMT expression. The relationship between analgesic-induced attenuation of PGE₂ signaling and its potential epigenetic consequences in GC, raises the possibility for similar GC effects of common environmental exposures given that DDT/other pesticides (Liu et al. 2012; Wrobel et al. 2015), phthalates (Wang et al. 2010; Romani et al. 2014), bisphenols (Kim et al. 2010), and brominated flame retardants (Park and Loch-Carusio 2015) have all been shown to impact PGE₂ production in reproductive tissues.

Our findings provide circumstantial support for the possibility that analgesics might induce epigenetic changes in fetal GC in human pregnancy. Because this possibility cannot be evaluated in human pregnancy, our different model systems could be used to identify what specific epigenomic changes are induced in fetal human GC or NTera2 cells. PGE₂ appears to play a consistent role in modulating key components of the epigenetic regulatory machinery in fetal gonads and in other tissues (Arosh et al. 2015; Venza et al. 2012), as well as in numerous cancer cell types (Venza et al. 2012; Xia et al. 2012), so it may play a similar role in fetal GC.

Our study has limitations. Although the xenograft and culture models are relevant to some aspects of normal human fetal development, including GC proliferation and differentiation (Jørgensen et al. 2015; Mitchell et al. 2010; Spade et al. 2014), none of the models recreate *in vivo* human conditions. We used exposures believed to be consistent with therapeutic analgesic exposures in humans, but we were unable to perform dose-response studies due to limited availability of human fetal gonadal tissue; this should be an important focus of future studies. Although NTera2 cells derive from TGCC precursor cells, which are believed to originate from fetal GC (Hoei-Hansen et al. 2004), they are tumor cells, and not a true fetal GC line. Despite these limitations, the consistency of the effects of analgesics on GC/NTera2 cell numbers and their concordance between humans and rats, including *in vivo* in the latter (Dean et al. 2016), suggest that the mechanisms involved in analgesic/PGE₂ effects on fetal GC are conserved between rodents and humans.

In conclusion, the present data suggest that exposure of human fetal ovaries or testes to therapeutically relevant concentrations of acetaminophen and ibuprofen, that reflect serum levels measured in humans following a therapeutic dose of these medications, causes a consistent decrease in fetal GC number in addition to effects on gene expression and, potentially, epigenetic modifications. These effects are highly reproducible, conserved in rat and human across different model systems, and probably result from disruption of PGE₂ action. Although translation of our results to human pregnancy has to be considered with caution, they add to a growing body of evidence concerning potential effects of analgesics during pregnancy on human developmental outcomes (Hurtado-Gonzalez and Mitchell 2017; Kristensen et al. 2011; Lind et al. 2017; Mazaud-Guittot et al. 2013; van den Driesche et al. 2015). This, coupled with the high prevalence of analgesic use during pregnancy, suggest that prospective studies to investigate potential effects of analgesic use in pregnancy on outcomes in offspring should be a research priority. Furthermore, the potential reproductive/GC effects of analgesic exposure need to be taken into account in future epidemiological studies associating chemical exposures in pregnancy and reproductive outcomes in children.

Acknowledgments

P.H.G., R.M.S., R.A.A., and R.T.M. conceived and designed the experiments. P.H.G., S.v.D., J.McD., A.J., C.McK., S.Mcp., and K.K. performed the experiments. P.H.G., S.v.D., R.T.M., J. McD., and R.M.S. analyzed the data. R.T.M., R.M.S., and R.A.A. contributed reagents/materials/analysis tools. P.H.G., R.M.S., R.T.M., and R.A.A. wrote the paper. All authors approved the submitted version.

We gratefully acknowledge the work of A. Saunderson, R. Bayne, R. Rosario, and the staff of the Bruntsfield Suite of the Royal Infirmary of Edinburgh and the Human Developmental Biology Resource (<http://www.hdbr.org>) for providing tissue for these studies (Joint MRC/Wellcome Trust; grant 099175/Z/12/Z). We also acknowledge W. Mungall, J. Henderson, and M. Dodds for assistance with the animal work and tissue collection.

This project was funded by grants G33253 and G0901839 from the UK Medical Research Council (R.M.S. and R.A.A.), and by a Wellcome Trust Intermediate Clinical Fellowship (grant 098522) and a British Society of Paediatric Endocrinology and Diabetes (BSPED) research award (R.T.M.). The funding bodies have had no input into the conduct of the research or the production of this manuscript.

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